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TBC1D8B loss-of-function mutations lead to X-linked nephrotic syndrome via defective trafficking pathways --Manuscript Draft--

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Abstract:	<p>Steroid-resistant nephrotic syndrome (SRNS) is characterized by high range proteinuria and most often focal and segmental glomerulosclerosis (FSGS). Identification of mutations in genes causing SRNS has improved our understanding of disease mechanisms and highlighted defects in the podocyte, a highly specialized glomerular epithelial cell, as major factors in disease pathogenesis. By exome sequencing, we identified missense mutations in TBC1D8B in two families with an X-linked early-onset SRNS with FSGS. TBC1D8B is an uncharacterized Rab-GTPase activating protein likely involved in endocytic and recycling pathways. Immunofluorescence studies revealed TBC1D8B presence in human glomeruli, and affected individual podocytes displayed architectural changes associated with migration defects commonly found in FSGS. In zebrafish we demonstrated that both knockdown and knockout of the unique TBC1D8B ortholog induced proteinuria and that this phenotype was rescued by human TBC1D8B mRNA injection, but not by any of the 2 mutated mRNAs. We also showed an interaction between TBC1D8B and Rab11b, a key protein in vesicular recycling in cells. Interestingly, both internalization and recycling processes were dramatically decreased in affected individuals' podocytes and fibroblasts confirming the crucial role of TBC1D8B in the cellular recycling processes, probably as a Rab11b GTPase activating protein. Altogether, these results confirmed that pathogenic variations in TBC1D8B are involved in X-linked podocytopathy and points to alterations in recycling processes as a mechanism of SRNS.</p>

TBC1D8B loss-of-function mutations lead to X-linked nephrotic syndrome via defective trafficking pathways

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ABSTRACT (218/250 words)

Steroid-resistant nephrotic syndrome (SRNS) is characterized by high range proteinuria and most often focal and segmental glomerulosclerosis (FSGS). Identification of mutations in genes causing SRNS has improved our understanding of disease mechanisms and highlighted defects in the podocyte, a highly specialized glomerular epithelial cell, as major factors in disease pathogenesis. By exome sequencing, we identified missense mutations in *TBC1D8B* in two families with an X-linked early-onset SRNS with FSGS. *TBC1D8B* is an uncharacterized Rab-GTPase activating protein likely involved in endocytic and recycling pathways. Immunofluorescence studies revealed *TBC1D8B* presence in human glomeruli, and affected individual podocytes displayed architectural changes associated with migration defects commonly found in FSGS. In zebrafish we demonstrated that both knockdown and knockout of the unique *TBC1D8B* ortholog induced proteinuria and that this phenotype was rescued by human *TBC1D8B* mRNA injection, but not by any of the 2 mutated mRNAs. We also showed an interaction between *TBC1D8B* and Rab11b, a key protein in vesicular recycling in cells. Interestingly, both internalization and recycling processes were dramatically decreased in affected individuals' podocytes and fibroblasts confirming the crucial role of *TBC1D8B* in the cellular recycling processes, probably as a Rab11b GTPase activating protein. Altogether, these results confirmed that pathogenic variations in *TBC1D8B* are involved in X-linked podocytopathy and points to alterations in recycling processes as a mechanism of SRNS.

Main Text

Steroid-resistant nephrotic syndrome (SRNS) is a glomerular disease characterized by massive proteinuria, most often associated with focal and segmental glomerulosclerosis (FSGS)¹. SRNS is responsible for chronic kidney disease and accounts for 15% of end-stage kidney disease (ESKD) cases in individuals under 25 years of age². In glomeruli, podocytes are terminally differentiated, highly specialized epithelial cells. Podocyte foot processes (FP) wrap the outer surface of the glomerular capillaries and interdigitate with FP of neighboring podocytes forming specialized cell-cell junctions called slit-diaphragms (SD), a major constituent of the glomerular filtration barrier. Identification of monogenic causes of SRNS involving more than forty genes has helped decipher podocyte physiology^{3; 4}. Many studies have reported a strong relationship between the SD and the actin cytoskeleton⁴, and the role of vesicular trafficking has been well established in the maintenance of SD complexes^{5; 6}. Nevertheless, intracellular transport defects have rarely been related to monogenic SRNS⁷.

Now, through studies aiming at identifying new genes mutated in SRNS in two families, we identified a potential actor of endosomal trafficking. The first family (family A) was from Ecuador and presented with isolated congenital SRNS consistent with an X-linked inheritance. Pedigree and clinical features are described in Figure 1A and Table 1. Briefly, affected females (I-2 and II-2) exhibited non-nephrotic proteinuria, while affected boys (II-1; II-3 and II-4) developed congenital or early onset NS. All pregnancies were marked by pre-eclampsia when carrying a male boy. In the proband (II-4) and her sister (II-2), kidney biopsy revealed FSGS lesions, and electron microscopy (EM) showed FP effacement, a hallmark of NS¹. The proband (II-4) reached ESKD by the age of 2.8y and did not exhibit recurrence after kidney transplantation. By exome sequencing (ES), we identified a c.738G>C variant in *TBC1D8B*

(RefSeq NM_198881.1; HGNC:24715) localized on chromosome X (Figure 1B). This missense variant (p.Gln246His) was defined as probably damaging by pathogenicity prediction softwares. Through a European collaborative network (Eurenomics), we identified another sporadic SRNS affected individual (II-2) in a family (family B) originated from the UK Renal Rare Disease Registry, (RaDaR #425), with a missense c.872T>C (p.Phe291Ser) variant in the same gene (Figure 1C-D – Table 1). This variant also displayed highly pathogenic scores. The affected individual (II-2), a male from European ancestry presenting with early onset SRNS at the age of 2 years, had no other systemic features. Kidney biopsy revealed global sclerosis on light microscopy and FP effacement on EM (Figure 1E). He reached ESKD by the age of 9 years and underwent kidney transplantation a year later. Both mutations segregated with the affected status in the respective families and were respectively present in 1/27314 (with no hemizygous) and absent from gnomAD database in the population-matched controls. Both mutations involved residues conserved from *C. elegans* to *H. sapiens* (Figure S1A-B).

TBC1D8B is a member of the TBC domain protein family (Tre-2/Bub2/CDC16). Like other TBC proteins, TBC1D8B may function as a Rab-GTPase Activating Protein (Rab-GAP) by binding to specific Rab proteins and stimulating their GTPase activity⁸. Interestingly, in addition to its Rab-GAP (TBC) domain, TBC1D8B contains one GRAM domain repeated two times, which allows binding to lipid rafts, critical elements of SD signaling in podocytes⁹. The two mutations described above are localized within each GRAM domain (Figure 1F). TBC1D8B also possesses an EF-Hand domain localized at the C-terminal extremity. A second isoform of TBC1D8B results from alternative splicing events in intron 11, and leads to a shorter 632 amino-acid protein, lacking the C-terminal extremity of the Rab-GAP domain and the entire

EF-like domain. However, the catalytic residues (Arg534 and Glu574) are present in both protein isoforms.

The role of TBC1D8B has never been explored either in cell lines or animal models. Immunofluorescence studies in human kidneys revealed the presence of TBC1D8B both in glomerular podocytes and tubules at early stages (25 weeks) and in mature kidney. In glomeruli, TBC1D8B colocalized with synaptopodin a specific cytosolic protein¹⁰ (Figure 2A). To further comprehend the role of TBC1D8B, we undertook functional studies in both zebrafish and human podocytes. Podocytes from individual B (harboring the p.Phe291Ser variant) were conditionally immortalized from the affected individual's nephrectomy specimen using our published methodology¹¹. As previously described in some monogenic SRNS, podocytes often display alterations in actin cytoskeleton organization¹²⁻¹⁶ associated with changes in adhesion and migration^{14; 17}. Podocytes from affected individual B exhibited similar phenotypes, and TBC1D8B displayed an intracellular vesicular localization as shown in figure 2B-D. Podocytes from individual A were not available, since he underwent bilateral nephrectomy several years before.

According to the Zebrafish Genome Reference Consortium (GRCz11), *tbc1d8b* is the only zebrafish ortholog and is located on autosome 5. Only one transcript has been described corresponding to the longer isoform in humans. By whole-mount *in situ* hybridization in 24 post-fertilization (hpf) zebrafish embryos, we demonstrated *tbc1d8b* expression in the neural tube, brain, pectoral fins, and the pronephric glomerulus (Figure S2). Functional analyses were then performed in both *tbc1d8b* knock-down (KD) morphants and knock-out (KO) fish. Morphants were obtained by specifically targeting *tbc1d8b* with either splice-blocking (MO^{SPLICE}) or translation-blocking (MO^{ATG}) morpholino oligonucleotides. The efficacy

of MO^{SPLICE} was checked by RT-PCR (Figure S3A), and the efficacy of MO^{ATG} was demonstrated by the absence of fluorescence after co-injection of the MO^{ATG} with its *tbc1d8b* 5'UTR mRNA target fused to the GFP as described in Figure S3B. The *tbc1d8b*^{-/-} mutant fish line was obtained by ENU mutagenesis resulting in a nonsense mutation in exon 9 (European Zebrafish Resource Center - EZRC). The final product is a truncated 468 amino-acid protein missing the Rab-GAP domain (which starts at residue 466). In zebrafish, pericardial edema has been previously reported to be an indirect marker of a glomerular filtration barrier defect and proteinuria¹⁶, although it is not specific and can be seen in other zebrafish abnormalities (i.e. heart defects, global developmental abnormalities). Such a phenotype was observed in >95% of both MO^{SPLICE} and MO^{ATG} morphants at 96hpf whereas it was found in less than 2% of control MO fish at 96hpf (Figure 3A-B). An identical phenotype was obtained in the *tbc1d8b*^{-/-} fish with the expected Mendelian ratio (n=261 - 22.9% phenotype in *tbc1d8b*^{+/-} offspring) - Figure 3C).

To characterize the effect of *tbc1d8b* loss on kidney anatomy, histology was performed and showed a retracted glomerulus in an enlarged Bowman's capsule in *tbc1d8b*^{-/-} fish compared to control (Figure 3D). Similar to affected individuals, EM revealed significant FP effacement and disappearance of SD in *tbc1d8b*^{-/-} KO fish compared to control (number of FP per μm 3.5 ± 0.2 and 5.3 ± 0.2 respectively - $p < 0.0001$) as indicated in Figure 3E and S4.

To confirm the glomerular permeability defect, we set-up a dye-filtration assay. A high molecular-weight FITC-labeled dextran (500kDa) was injected in the cardinal vein of MO^{SPLICE} and MO^{CONTROL} and *tbc1d8b*^{-/-} or *tbc1d8b*^{WT} zebrafish at 96hpf. We then screened the filtration defects in two different ways. We first measured fluorescence in the retinal vein and showed that fluorescence in the vasculature of MO^{SPLICE} fish was significantly decreased

compared to MO^{CONTROL} (Figure S5). We then showed specific glomerular leakage by imaging dye uptake in tubular cells 6 hours-post-injection (6hpi). Fluorescence was observed in tubular cells in KO *tbc1d8b*^{-/-} fish, but not in control fish, revealing a glomerular protein leakage consistent with pericardial edema and EM findings (Figure 3F).

To investigate the functional consequences of both mutations, we performed rescue experiments in zebrafish using the human short isoform of WT and mutant mRNAs, since in wild-type human cultured podocytes, the 632 amino-acid short isoform seems to be predominantly expressed (Figure S6). As mentioned previously, edema was described in 97.6% of morphants. However, when mRNA^{WT} (100pg) was co-injected with MO, this ratio fell to 16% (n=148) (Figure 3G) with a dose-response effect (Figure S7). Conversely, the mutated mRNA^{p.Gln246His} and mRNA^{p.Phe291Ser} were only able to very partially rescue the phenotype with 80% (n=201) and 68% (n=282) of fish exhibiting pericardial edema respectively (Figure 3G). These results indicate that although both mutations are clearly damaging in both individuals, a modest protein residual activity is likely.

As mentioned above, TBC1D8B may act as Rab-GAP, promoting GTP hydrolysis in specific Rab proteins¹⁸. In mammalian cells including podocytes, Rab-GTPases are mostly involved in vesicular trafficking^{19; 20}. To further analyze the role of TBC1D8B in this process, we performed transferrin endocytosis and recycling assays in affected individuals' podocytes and fibroblasts harboring p.Phe291Ser and p.Gln246His variants respectively. Transferrin is a widely used marker for both clathrin-mediated internalization and recycling pathways in most cell types. After internalization from the plasma membrane and before being recycled back to cell surface, transferrin and its receptor accumulate in the perinuclear recycling compartment (PNRC)^{21; 22}. We detected a delay in transferrin uptake in affected individuals'

cells compared to control. After 15 and 60 min of incubation (in fibroblasts and podocytes respectively) most of the fluorescence was found in the PNRC of WT cells. However, endocytosis was significantly slower in mutant fibroblasts and podocytes (Figure 4A-B and Figure S8A-B). In addition, analysis of transferrin recycling revealed a significant decrease of normalized fluorescence intensity 45min after transferrin loading in WT podocytes as well as in fibroblasts, whereas it was maintained in the PNRC in affected individuals' cells (Figure 4C-D and Figure S8C-D). Altogether, these results strongly suggest that both endocytosis and vesicular recycling are altered in affected podocytes.

In cells, PNRC dynamics and recycling are mostly regulated by Rab11. We then hypothesized that TBC1D8B might be a crucial GAP for Rab11 that displays at least two isoforms (Rab11a and Rab11b)²² both present in mouse podocytes²³. By co-immunoprecipitation experiments in HEK293T cells, we showed that the Rab-binding domain (RBD) of TBC1D8B was able to interact with a dominant active, GTP-bound, mutant of Rab11b (DA-Rab11b), but not with DA-Rab11a (Figure 4E-F). We also observed a specific interaction between endogenous TBC1D8B and DA-Rab11b (Figure 4G). In addition, we showed that DA-Rab11b and TBC1D8B Rab-binding domain colocalized at the PNRC when co-expressed in podocytes (Figure S9), but interestingly, the widespread Rab11b signal in WT cells shifted to a very restricted localization to the PNRC in mutant podocytes, which suggests that GTP-bound Rab11b is trapped in the PNRC in cells from affected individual. (Figure S10A-B). Altogether, these data strongly suggest that TBC1D8B is a specific Rab11b-GTPase activating protein.

Numerous proteins are subject to Rab11-dependent recycling in various cell types, especially in neurons²⁴. However, although it has been shown that some proteins are

recycled in a Rab11b-dependent manner²⁵, only a few studies explored the role of Rab11b on vesicular trafficking. In 2016, Grimsey *et al.* reported the crucial role of Rab11b GTP hydrolysis for the initiation of transferrin recycling from the PNRC to the plasma membrane²⁶. In podocytes SD integrity is of utmost importance and several SD proteins are subjected to endocytic and recycling events to maintain their regulation²⁷. Very recently, mutations in *GAPVD1*, a Rab5 effector showed to be involved in nephrin regulation at the SD, were reported as disease-causing²⁸. In our study, we did not find any interaction between TBC1D8B and Rab5 (Figure 4F), suggesting that Rab5 is not a target of TBC1D8B. Thus, our results highly suggest that some podocyte specific proteins could specifically use the Rab11b-dependent recycling pathway, as it has been shown for some SD proteins and the use of the Rab5-dependent pathway²⁸. By this means, alterations in Rab11b activation would lead to SD dysregulation, implying that not only endocytosis, but also recycling are of fundamental importance for SD integrity. Indeed, it is likely that mutations in *TBC1D8B* lead to strong defect in recycling processes in podocytes, that could also induce a defect in transferrin uptake linked to decrease number of receptors at the plasma membrane at steady state.

Altogether, our results confirmed that mutations in *TBC1D8B* are involved in the development of early SRNS in rare affected individuals. We show herein that vesicular trafficking plays a fundamental role in podocyte disease, especially in SRNS, through a Rab11-dependent recycling process. Recent discoveries in monogenic SRNS and especially in SD regulation during disease through endocytic and recycling pathways would certainly help to develop targeted therapy for affected individuals.

Supplemental data

Supplemental contains 11 figures, 1 table and supplemental material and Methods.

Figure S1: TBC1D8B amino-acid conservation

Figure S2: *tbc1d8b* *In situ* hybridization in zebrafish

Figure S3A-B: Verification of both initiation blocking and splice MO specificity.

Figure S4: Electron microscopy of zebrafish glomeruli

Figure S5: Dye filtration eye assay in zebrafish

Figure S6: TBC1D8B expression in cultured podocytes and HEK293 cells

Figure S7: Dose response effect of *TBC1D8B* human mRNA injection in morphants zebrafish

Figure S8A-D: Transferrin assay in mutated fibroblasts and control

Figure S9: Cellular localization of DA-Rab11b and the Rab-Binding-Domain (RBD) of TBC1D8B in podocytes

Figure S10A-B: Cellular localization of endogenous Rab11b

Figure S11 – Immunofluorescence staining of cellular compartment in affected individuals and controls

Table S1: Primers

Methods

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Declaration of interests

The authors declare no competing interests.

Web Resources :

The URLs for data presented herein are as follows:

Eurenomics: <https://eurenomics.eu/>

gnomAD: <http://gnomad.broadinstitute.org/>

PolyPhen : <http://genetics.bwh.harvard.edu>

SIFT <http://sift.jcvi.org>

Mutation taster : <http://www.mutationtaster.org/>

Praline : <http://www.ibi.vu.nl/programs/pralinewww/>

Online Mendelian Inheritance in Man: <http://www.omim.org>

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Figure legends

Figure 1 – Clinical and genetic information for families A and B

(A-C) Family pedigrees for families A (on the left) and B (on the right). In family A, the proband (II-4) and his brothers (II-1 and II-3) developed congenital nephrotic syndrome while individual from family B (II-2) developed an early-onset nephrotic syndrome. (Arrows indicate probands). *TBC1D8B* mutations c.738G>C and c.872T>C were detected in affected and carriers of families A and B respectively.

(B-D) Exome sequencing revealed two distinct missense variants in *TBC1D8B* (c.738G>C and c.872T>C). Segregation of variants was confirmed by Sanger sequencing in both families.

(E) Electron microscopy image from affected individual from family B (II-2) harboring the p.Phe291Ser mutation revealed foot processes effacement (white arrowheads) (scale-bar 2µm)

(F) *TBC1D8B* protein exhibits two isoforms that include both mutations. Mutations localized in Glucosyltransferases, Rab-like GTPase activators and Myotubularins (GRAM) domains at the N-terminal extremity. Catalytic residues on the Rab-GTPase activating (GAP) domain are maintained. C-terminal EF-hand domain is not conserved in isoform 2. EF-hand domain has been described in other proteins as a calcium-binding regulation site, which may downregulate the protein activity when bound to Ca²⁺ 29.

Figure 2 - TBC1D8B localization and phenotype observed in mutated podocytes

(A) Immunofluorescence studies were performed on human fetal kidney at 25 gestational weeks (top panel) and in mature kidney in a healthy 7-year-old male (bottom panel). TBC1D8B displayed a glomerular localization with partial colocalization with synaptopodin, a cytosolic podocyte protein. (Scale bar 20µm)

(B) In immunofluorescence experiments in immortalized podocytes, TBC1D8B displayed an intracellular vesicular expression. F-actin cytoskeleton was detected by immunofluorescence with phalloidin staining. p.Phe291Ser human mutant podocytes cortically (arrowhead) reorganized their F-actin cytoskeleton. Original magnification x63.

(C) Cell adhesion was measured with a spectrophotometer at 570 nm optical density (n=3, **p< 0.01, mean ±SEM).

(D) Human podocyte cell motility and migration were measured by scratch assays. Number of cells per unit was significantly higher in the WT group 12 hr after the scratch as shown on the graph on right (n=3, ***p < 0.0001, mean ±SEM).

Figure 3 - Phenotype observed in *tbc1d8b* KO and KD zebrafish

(A) KO fish displayed pericardial edema at 48hpf on mutant fish (black arrow). Scale bar 1mm.

(B) Both MO^{ATG} and MO^{SPLICE} also exhibited pericardial edema in more than 95% fish whereas MO^{CONTROL} did not. (n>200, *** p<0.0001, Mean ± SEM)

(C) *tbc1d8b*^{+/-} offspring displayed pericardial edema with a Mendelian ratio.

(D) Regular optical microscopy revealed a retracted glomerulus (*) in an enlarged Bowman's capsule (white circle) in mutated *tbc1d8b*^{+/-} fish compared to control (scale bar 15µm).

(E) Electron microscopy findings showed a mixture of foot processes (FP) effacement (*) with regular FP (black arrowheads). Graph on the right shows a significantly lower rate of FP per μm in *tbc1d8b*^{-/-} fish compared to control (n= 3 fish per condition, ***p<0.001, mean \pm SEM).

(F) Dye filtration assay was performed in KO and control fish. While Texas-Red 10kDa fluorescence uptake was physiologically found in both *tbc1d8b*^{-/-} and ^{+/-}, FITC 500kDa fluorescence was only found in KO fish (n=3). (Asterisk represents tubular lumen) (Scale bar 5 μm)

(G) In rescue experiments, only human WT mRNA was able to significantly decrease pericardial edema whereas both mutated mRNA were only able to partially reduce phenotype in fish (n> 100 / condition).

Figure 4 - TBC1D8B interaction and effect of mutations on vesicular trafficking

(A-B) Transferrin uptake is evaluated by incubation with fluorescently labeled transferrin. Fluorescence intensity progressively increased in control podocytes whereas it increased much more slowly in mutated podocytes harboring the p.Phe291Ser mutation suggesting an endocytosis defect. Graph B represents the quantification of normalized fluorescence in both mutated and control cells (*** p<0.001, mean \pm SEM). (Scale bar 25 μm)

(C-D) Transferrin chase evaluates the recycling process in podocytes. Measuring recycling of fluorescently labeled transferrin in WT and mutant podocytes revealed persistence of fluorescence 45 min after internalization in mutant cells while it reached 50% of T0 intensity in control podocytes, indicating a delay in recycling processes in mutant cells (C). Graph D represents the quantification of normalized fluorescence in both mutated and control cells (*** p<0.001 at 45 min, mean \pm SEM). (Scale bar 25 μm)

(E) Construct used for to perform co-immunoprecipitation with a specific plasmid harboring only the TBC1D8B Rab-binding domain (RBD) tagged with the myc epitope, and under the CMV promoter.

(F) In co-transfected HEK293T, we showed that the (myc-)RBD of TBC1D8B was able to interact with Dominant Active (DA)-Rab11b but not DA-Rab11A nor DA-Rab5.

(G) The endogenous TBC1D8B protein interacts with transfected DA-Rab11b-GFP in HEK cells.

Table 1 - Mutations in *TBC1D8B* in two families with a likely X-linked FSGS

Family	Individual	Ethnic origin	Sex	Nucleotide alteration	Exon	Status	Protein alteration	Polyphen / Sift/Mutation taster	Amino-acid conservation	Age at proteinuria onset	Immuno-sensitivity	Pathology	Age at ESRD	Age at transplantation	Recidive	Death
A	NCR2230 Index	Ecuador	M	c.738G>C	5	Hemi	p.Gln246 His	0.996/0/DC	<i>C.elegans</i>	Congenital	No	Collapsing FSGS	2.8yrs	4yrs (Thrombosis) 7yrs	No	N/A
	NCR1387 Mother	Ecuador	F			Hetero				Adult	N/A	N/A		N/A	N/A	N/A
	NCR1388 Sister	Ecuador	F			Hetero				7yrs	No	FSGS	No	N/A	N/A	N/A
	Brother 1	Ecuador	M	N/A	N/A	N/A	N/A	N/A	N/A	Congenital	No	N/A	N/A	N/A	N/A	4 mo
	Brother 2	Ecuador	M	N/A	N/A	N/A	N/A	N/A	N/A	5 months	No	N/A	N/A	N/A	N/A	14 mo
B	104	European	M	c.872T>C	6	Hemi	p.Phe291 Ser	0.961/0/DC	<i>C.elegans</i>	2 yrs	No	Chronic progressive sclerosis	9 yrs	10 yrs	No	N/A

Abbreviations: M: Male; F: Female; yrs: years; mo: months; FSGS: Focal and Segmental glomerulosclerosis; Hemi: hemizygous; Hetero: Heterozygous, N/A: Non Applicable, ESRD: End Stage Renal Disease







